

Human factor Va1 and factor Va2

Citation for published version (APA):

Hoekema, L., Nicolaes, G. A. F., Hemker, H. C., Tans, G., & Rosing, J. (1997). Human factor Va₁ and factor Va₂: Properties in the procoagulant and anticoagulant pathways. *Biochemistry*, 36(11), 3331-3335. <https://doi.org/10.1021/bi9623284>

Document status and date:

Published: 18/03/1997

DOI:

[10.1021/bi9623284](https://doi.org/10.1021/bi9623284)

Document Version:

Publisher's PDF, also known as Version of record

Please check the document version of this publication:

- A submitted manuscript is the version of the article upon submission and before peer-review. There can be important differences between the submitted version and the official published version of record. People interested in the research are advised to contact the author for the final version of the publication, or visit the DOI to the publisher's website.
- The final author version and the galley proof are versions of the publication after peer review.
- The final published version features the final layout of the paper including the volume, issue and page numbers.

[Link to publication](#)

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal.

If the publication is distributed under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license above, please follow below link for the End User Agreement:

www.umlib.nl/taverne-license

Take down policy

If you believe that this document breaches copyright please contact us at:

repository@maastrichtuniversity.nl

providing details and we will investigate your claim.

Human Factor Va₁ and Factor Va₂: Properties in the Procoagulant and Anticoagulant Pathways[†]

Lico Hoekema, Gerry A. F. Nicolaes, H. Coenraad Hemker, Guido Tans, and Jan Rosing*

Cardiovascular Research Institute Maastricht, Maastricht University, Maastricht, The Netherlands

Received September 16, 1996; Revised Manuscript Received December 31, 1996[®]

ABSTRACT: Human plasma factor V is heterogeneous and yields two forms of activated factor V that bind with low (factor Va₁) and high affinity (factor Va₂) to phospholipids. The properties of factor Va₁ and factor Va₂ in the anticoagulant and procoagulant pathways were evaluated by comparing their sensitivity for inactivation by APC and their ability to act as cofactor in prothrombin activation. At low phospholipid concentrations and on membranes containing low amounts of phosphatidylserine (PS), factor Va₁ was inactivated by APC at 15-fold lower rates than factor Va₂, both in the absence and in the presence of protein S. At high phospholipid concentrations and on membranes with more than 15 mol % PS, factor Va₁ and factor Va₂ were inactivated with equal efficiency. Differences between cofactor activities of factor Va₁ and factor Va₂ in prothrombin activation were only observed on membranes with less than 7.5 mol % PS. Due to the different phospholipid requirements of APC-catalyzed factor Va inactivation and of expression of factor Va cofactor activity in prothrombin activation, the thrombin-forming capacity of factor V₁ was 7-fold higher than that of factor V₂ in a reaction system containing factor Xa, prothrombin, APC, protein S, vesicles with a phospholipid composition resembling that of activated platelets, and traces of thrombin to initiate prothrombin activation. This shows that in the process of generation, expression, and down-regulation of factor Va cofactor activity on physiological membranes, the overall procoagulant activity of factor V₁ can considerably exceed that of factor V₂.

Inactivation of factor Va by activated protein C (APC)¹ plays an important role in the down-regulation of thrombin formation (Esmon, 1989). Factor Va loses its cofactor activity in prothrombin activation after APC-catalyzed cleavage in its heavy-chain domain at positions Arg⁵⁰⁶ and Arg³⁰⁶ (Kalafatis *et al.*, 1994, 1995; Nicolaes *et al.*, 1995). Factor Va inactivation by APC is stimulated by negatively charged phospholipids (Walker *et al.*, 1979; Bakker *et al.*, 1992) and protein S (Bakker *et al.*, 1992; Walker, 1980; Solymoss *et al.*, 1988). Recently, we reported that protein S promotes factor Va inactivation by specifically accelerating the slow peptide bond cleavage at Arg³⁰⁶ (Rosing *et al.*, 1995).

The physiological importance of APC and protein S is demonstrated by the association of venous thrombosis with hereditary deficiencies in protein C (Griffin *et al.*, 1981) or protein S (Schwarz *et al.*, 1984; Comp *et al.*, 1984) and by the occurrence of familial thrombophilia in individuals that have a hereditary defect (replacement of Arg⁵⁰⁶ by Gln) that makes factor Va insensitive to proteolytic inactivation by APC (APC resistance, Dahlbäck *et al.*, 1993; Bertina *et al.*, 1994).

In 1993 we reported (Rosing *et al.*, 1993) that plasma contains two forms of factor V which likely differ in the degree of glycosylation in the carboxy-terminal C2 domain (Ortel *et al.*, 1994a). Activation by thrombin yields two forms of factor Va (factor Va₁ and factor Va₂) with different affinities for procoagulant membrane surfaces and which exhibit different cofactor activities in prothrombin activation. Considering the physiological importance of down-regulation of factor Va activity via the anticoagulant pathway, we have compared the procoagulant and anticoagulant properties of factors Va₁ and Va₂ in order to get insight in their contributions to *in vivo* thrombin formation. We report that under several conditions factors Va₁ and Va₂ have similar procoagulant activity whereas they considerably differ in their sensitivity for APC. In these situations, the overall procoagulant activity of factor V₁ is higher than that of factor V₂.

EXPERIMENTAL PROCEDURES

Materials. Phospholipids were obtained from Avanti Polar Lipids, Pelham, AL. Small unilamellar phospholipid vesicles were prepared as described before (Rosing *et al.*, 1993). S2238, S2366, and I2581 were supplied by Chromogenix, Mölndal, Sweden. Materials used for protein purification were purchased from Pharmacia. Human prothrombin, thrombin, and factor Xa were prepared by earlier described procedures (DiScipio *et al.*, 1977; Pletcher & Nelsestuen, 1982; Bock *et al.*, 1989). Human protein C was prepared and activated as reported by Gruber *et al.* (1989). Human protein S was obtained from Enzyme Research Laboratories Inc. (Swansea, U.K.). Human factor V₁, factor V₂, factor Va₁, and factor Va₂ were purified as described previously (Rosing *et al.*, 1993).

Protein Concentrations. Molar prothrombin, thrombin, and factor Xa concentrations were determined as described

[†] This work was supported by Program Grant 900-526-192 from the Dutch Organisation for Scientific Research (NWO).

* To whom correspondence should be addressed at the Department of Biochemistry, Maastricht University, P.O. Box 616, 6200 MD Maastricht, The Netherlands. Telephone: 31-43-3881678. Fax: 31-43-3670988.

[®] Abstract published in *Advance ACS Abstracts*, March 1, 1997.

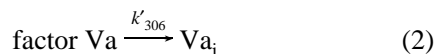
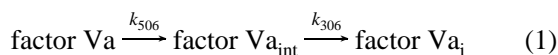
¹ Abbreviations: APC, activated protein C; BSA, bovine serum albumin; I2581, *N*-dansyl-(*p*-guanidino)phenylalaninepiperidide hydrochloride; S2238, D-Phe-(pipecolyl)-Arg-pNA; S2366, L-pyroGlu-Pro-Arg-pNA; PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phosphoserine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine.

before (Chase & Shaw, 1969; Smith, 1973). APC concentrations were determined with S2366 using kinetic parameters reported by Sala *et al.* (1984). Protein S concentrations were calculated from the A_{280} using an $A_{280}^{1\%}$ of 9.5 and $M_r = 70\,000$ for protein S (DiScipio & Davie, 1979). Factor Va was quantitated as described below.

Factor Va Assay. Cofactor activity of factor Va was quantitated by determining the rate of factor Xa-catalyzed prothrombin activation in reaction mixtures that contained a limiting amount of factor Va, 5 nM factor Xa, 50 μ M phospholipid vesicles (DOPS/DOPC, 10/90, mol/mol), and 0.5 μ M prothrombin (Tans *et al.*, 1991a). Rates of prothrombin activation were determined with S2238 (Rosing *et al.*, 1980). The molar factor Va concentration in the assay mixture was calculated from the rate of prothrombin activation using a turnover number of 6000 mol of prothrombin activated per minute per mole of factor Xa–Va complex (Tans *et al.*, 1991b).

Inactivation of Factor Va by APC. Factor Va₁ or factor Va₂ was incubated at 37 °C with phospholipid vesicles in the absence or presence of protein S in a reaction mixture containing 25 mM Hepes (pH 7.5), 175 mM NaCl, 3 mM CaCl₂, and 5 mg/mL BSA, and its concentration was determined in triplicate to obtain $[\text{factor Va}]_{t=0}$. Factor Va inactivation was started by addition of APC, and the time-dependent loss of factor Va cofactor activity was followed as described in the previous paragraph.

Curve Fitting of Pseudo-First Order Time Courses of Factor Va Inactivation. Time courses of factor Va₁ or factor Va₂ inactivation by APC were determined by monitoring the loss of cofactor activity of factor Va as a function of time. Data reported in the literature (Kalafatis *et al.*, 1994, 1995; Nicolaes *et al.*, 1995; Rosing *et al.*, 1995) demonstrate that the loss of factor Va cofactor activity can proceed via two pathways:



in which factor Va_{int} is a reaction intermediate with partial cofactor activity, factor Va_i is a form of factor Va that has completely lost its cofactor activity, k_{506} is the second-order rate constant for cleavage at Arg⁵⁰⁶, k_{306} is the second-order rate constant for cleavage at Arg³⁰⁶ in factor Va_{int}, and k'_{306} is the second-order rate constant for cleavage at Arg³⁰⁶ in native factor Va. The rate constants k_{506} , k_{306} , and k'_{306} and the cofactor activity of factor Va_{int} were obtained by fitting time courses of factor Va inactivation to eq 3 using nonlinear least-squares regression analysis (Nicolaes *et al.*, 1995; Rosing *et al.*, 1995).

$$[\text{Va}]_t = [\text{Va}]_0 e^{-(k_{506}+k'_{306})t} + B[\text{Va}]_0 \frac{k_{506}e^{-k_{306}t}}{k_{506} + k'_{306} - k_{306}} \times [1 - e^{-(k_{506}+k'_{306}-k_{306})t}] \quad (3)$$

In this equation, k_{306} , k'_{306} , and k_{506} are the rate constants for peptide bond cleavages defined above and B represents the residual cofactor activity (percent of native factor Va) of the reaction intermediate factor Va_{int}.

Cofactor Activity of Factor Va in Prothrombin Activation. Factor Va₁ or factor Va₂ was incubated with 2.5 pM factor Xa and phospholipid vesicles at 37 °C in 25 mM Hepes (pH

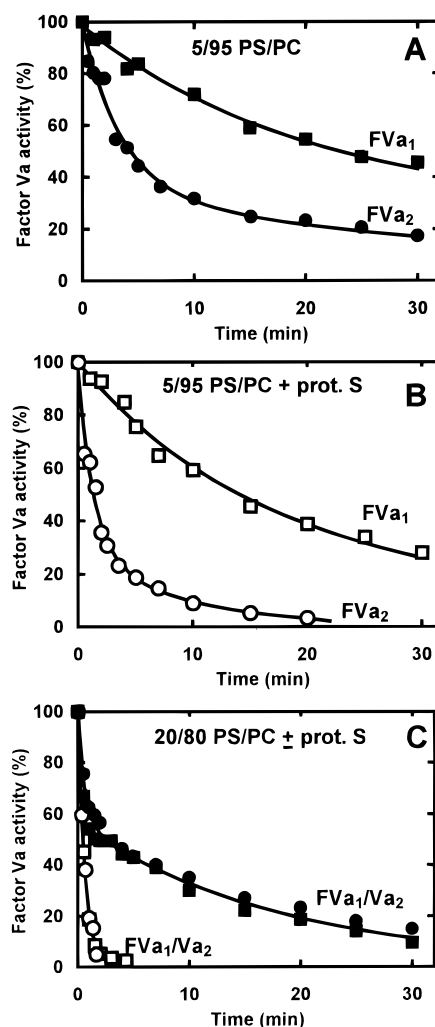


FIGURE 1: Inactivation of factor Va₁ and factor Va₂ by APC. 1.5 nM purified human factor Va₁ (■, □) or factor Va₂ (●, ○) was incubated at 37 °C with 1 nM APC (A, B) or 0.12 nM APC (C) without (■, ●) or with (□, ○) 200 nM protein S in 25 mM Hepes (pH 7.5), 175 mM NaCl, 3 mM CaCl₂, 5 mg/mL BSA, and (A,B) 5 μ M PS/PC (5/95, mol/mol) or (C) 50 μ M DOPS/DOPC (20/80, mol/mol). At the indicated time points, factor Va activity was determined as described under Experimental Procedures. The solid lines represent a best fit of the data to eq 3 given under Experimental Procedures using nonlinear least-squares regression analysis.

7.5), 175 mM NaCl, 3 mM CaCl₂, and 5 mg/mL BSA. After 5 min, prothrombin was added, and 2 and 4 min later, the amount of thrombin formed was quantitated as described before (Rosing *et al.*, 1980).

RESULTS

Effect of Phospholipid on Inactivation of Factor Va₁ and Factor Va₂ by APC in the Absence and Presence of Protein S. Recently, we have shown (Nicolaes *et al.*, 1995) that inactivation of factor Va by APC proceeds via a biphasic reaction (*cf.* Figure 1) in which fast cleavage at Arg⁵⁰⁶ in the heavy chain of factor Va results in the formation of an intermediate with partial cofactor activity in prothrombin activation that is fully inactivated by APC after slow cleavage at Arg³⁰⁶. We subsequently reported that protein S promotes factor Va inactivation by specifically accelerating cleavage at Arg³⁰⁶ (Rosing *et al.*, 1995).

It was also reported (Rosing *et al.*, 1993) that plasma factor V is heterogeneous and yields two forms of factor Va (Va₁ and Va₂) after activation that bind with different affinities to negatively charged membranes.

Table 1: Rate Constants ($M^{-1} s^{-1}$) for APC-Catalyzed Peptide Bond Cleavages in Factor Va₁ and Factor Va₂^a

| | no protein S | | with protein S | |
|--------------------------|-------------------|-------------------|-------------------|-------------------|
| | k_{506} | k_{306} | k_{506} | k_{306} |
| 5 μ M PS/PC (5/95) | | | | |
| factor Va ₁ | 8.3×10^5 | 3.4×10^4 | 1.1×10^6 | 5.7×10^5 |
| factor Va ₂ | 7.0×10^6 | 5.1×10^5 | 1.6×10^7 | 3.3×10^6 |
| 50 μ M PS/PC (20/80) | | | | |
| Factor Va ₁ | 2.1×10^8 | 6.4×10^6 | 2.2×10^8 | 1.2×10^8 |
| Factor Va ₂ | 1.2×10^8 | 6.6×10^6 | 1.2×10^8 | 1.4×10^8 |

^a The rate constants of APC-catalyzed inactivation of factor Va were obtained by fitting the time courses of factor Va inactivation (Figure 1) using the equation for a biphasic inactivation reaction described under Experimental Procedures.

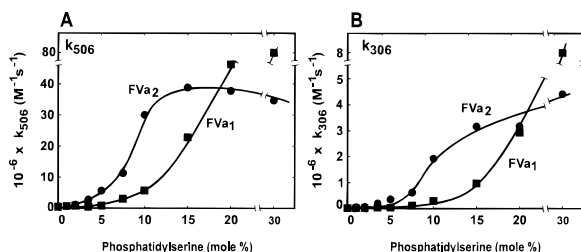


FIGURE 2: Effect of phosphatidylserine on rate constants of APC-catalyzed cleavage at Arg⁵⁰⁶ and Arg³⁰⁶ in factor Va₁ and factor Va₂. 1.5 nM purified human factor Va₁ (■) or factor Va₂ (●) was incubated at 37 °C with varying amounts (0.4–60 nM) of APC in 25 mM Hepes (pH 7.5), 175 mM NaCl, 3 mM CaCl₂, 5 mg/mL BSA, and 5 μ M DOPS/DOPC vesicles with the mole percentage phosphatidylserine (DOPS) indicated in the figure. Rate constants for APC-catalyzed cleavage at Arg⁵⁰⁶ (k_{506}) and Arg³⁰⁶ (k_{306}) were obtained from the best fit of the time courses of factor Va inactivation (cf. Figure 1) to eq 3 given under Experimental Procedures using nonlinear least-squares regression analysis. Plotted are k_{506} (A) and k_{306} (B) as a function of the phosphatidylserine content of the membrane.

In Figure 1, it is shown that on membranes with 5 mol % phosphatidylserine, factor Va₁ was inactivated by APC at considerably lower rates than factor Va₂, both in the absence (Figure 1A) and in the presence of protein S (Figure 1B). The rate constants for APC-catalyzed cleavage at Arg⁵⁰⁶ and Arg³⁰⁶ (k_{506} and k_{306}) were about 15 times lower for factor Va₁ than for factor Va₂ (Table 1). When the same experiment was performed in the presence of a 10-fold higher concentration of phospholipid vesicles containing 20 mol % phosphatidylserine, inactivation was much faster, and factor Va₁ and factor Va₂ were inactivated by APC at the same rate, both in the absence and in the presence of protein S (Figure 1C).

APC-Catalyzed Inactivation of Factor Va₁ and Factor Va₂ on Membranes Containing Varying Amounts of Phosphatidylserine. Time courses of factor Va₁ and factor Va₂ inactivation by APC (cf. Figure 1) were determined on 5 μ M phospholipid vesicles containing varying amounts of phosphatidylserine. Since protein S had similar effects on the inactivation of both forms of factor Va, we have performed these experiments in the absence of protein S. The time courses of factor Va inactivation were fitted using an equation for a biphasic inactivation reaction [eq 3; cf. Nicolaes *et al.* (1995) and Rosing *et al.* (1995)] which yielded the rate constants, k_{506} and k_{306} , for APC-catalyzed cleavage at Arg⁵⁰⁶ and Arg³⁰⁶ in the heavy chain of factor Va. Increasing the amount of phosphatidylserine in the membrane surface resulted in an increase of both k_{506} (Figure 2A) and k_{306} (Figure 2B) for each form of factor Va. This indicates

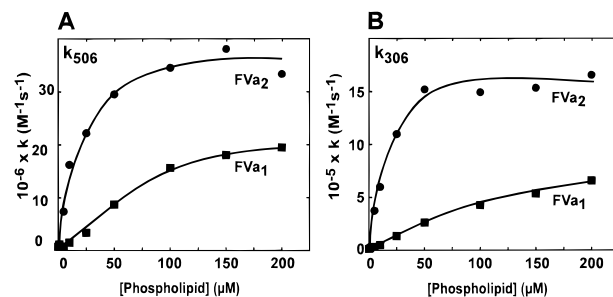


FIGURE 3: Effect of the phospholipid concentration on rate constants of APC-catalyzed cleavage at Arg⁵⁰⁶ and Arg³⁰⁶ in factor Va₁ and factor Va₂. 1.5 nM purified human factor Va₁ (■) or factor Va₂ (●) was incubated at 37 °C with varying amounts (2.6–46 nM) of APC in 25 mM Hepes (pH 7.5), 175 mM NaCl, 3 mM CaCl₂, 5 mg/mL BSA, and varying amounts of DOPS/DOPC (5/95, mol/mol). Rate constants for APC-catalyzed cleavage at Arg⁵⁰⁶ (k_{506}) and Arg³⁰⁶ (k_{306}) were obtained from the best fit of the time courses of factor Va inactivation (cf. Figure 1) to eq 3 given under Experimental Procedures using nonlinear least-squares regression analysis. Plotted are k_{506} (A) and k_{306} (B) as a function of the phospholipid concentration.

that the two cleavages required for full inactivation of factor Va are equally affected by variation of the phosphatidylserine content of the membrane. The data further show that factor Va₁ was inactivated at considerably lower rates than factor Va₂ on membranes with less than 15 mol % phosphatidylserine. At a higher phosphatidylserine content, both forms of factor Va were inactivated with similar rates, and at 30 mol % phosphatidylserine, factor Va₁ was even activated about 2 times faster than factor Va₂.

Effect of Variation of the Phospholipid Concentration on APC-Catalyzed Inactivation of Factor Va₁ and Factor Va₂. The previous experiments have shown that factor Va₁ was inactivated by APC at considerably lower rates than factor Va₂ on 5 μ M phospholipid vesicles containing 5 mol % phosphatidylserine. Since this likely reflects differences in the binding affinities of factors Va₁ and Va₂ for membrane surfaces (Rosing *et al.*, 1993), it is expected that the difference in sensitivity of both forms of factor Va for APC will annihilate at higher phospholipid concentrations. The experiment presented in Figure 3 shows that this indeed occurs. At low phospholipid concentrations, the rate constants, k_{506} (Figure 3A) and k_{306} (Figure 3B), for factor Va₁ inactivation were ~15-fold lower than those for factor Va₂. At increasing phospholipid concentrations, the rates of factor Va inactivation gradually converged, and at 200 μ M phospholipid, the difference between the rates of factor Va₁ and Va₂ inactivation was about 2-fold.

Effect of Phospholipid Composition and Concentration on the Cofactor Activity of Factor Va₁ and Factor Va₂ in Prothrombin Activation. In a previous paper (Rosing *et al.*, 1993), we reported that at low concentrations of phospholipid vesicles with a low phosphatidylserine content factor Va₂ exhibits a higher cofactor activity in prothrombin activation than factor Va₁. In Figure 4, it is shown, however, that optimal expression of factor Va cofactor activity in prothrombin activation required much less phospholipid and phosphatidylserine than APC-catalyzed factor Va inactivation (cf. Figures 2 and 3). Although at low phospholipid concentrations and on membranes with a low phosphatidylserine content factor Va₂ was a more effective cofactor than factor Va₁, the cofactor activities of both forms of factor Va were optimal and similar at 5 μ M phospholipid vesicles containing 10 mol % phosphatidylserine (Figure 4A) and

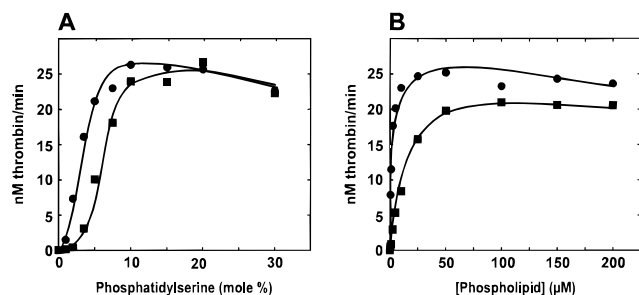


FIGURE 4: Effect of the phospholipid composition and concentration on the cofactor activity of factor Va₁ and factor Va₂ in prothrombin activation. 1.5 nM purified human factor Va₁ (■) or factor Va₂ (●) was incubated at 37 °C with 2.5 pM factor Xa in 25 mM Hepes (pH 7.5), 175 mM NaCl, 3 mM CaCl₂, 5 mg/mL BSA, and (A) 5 μM DOPS/DOPC vesicles with varying mole percentages of phosphatidylserine (DOPS) or (B) varying amounts of DOPS/DOPC (5/95, mol/mol) vesicles. After 5 min, prothrombin was added to a final concentration of 1 μM. The rate of prothrombin activation was determined as described under Experimental Procedures. Plotted are the rate of prothrombin activation as a function of the phosphatidylserine content of the membranes (A) or as a function of the phospholipid concentration (B).

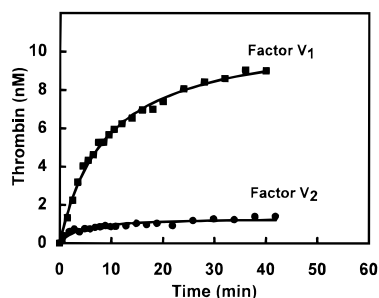


FIGURE 5: Comparison of procoagulant activities of factor V₁ and factor V₂ in a reaction system containing prothrombinase components, APC, and protein S. 1.5 nM purified human factor V₁ (■) or factor V₂ (●) was incubated at 37 °C with 0.1 nM thrombin, 4 pM factor Xa, 1 μM prothrombin, 2 nM APC, 200 nM protein S, and 12.5 μM vesicles containing 10% brain PS, 39% brain PC, 27% brain PE, 19% brain sphingomyelin, and 5% liver PI on a molar basis in 25 mM Hepes (pH 7.5), 127 mM NaCl, 3 mM CaCl₂, and 5 mg/mL BSA. The amounts of thrombin formed at the indicated time points were determined as described under Experimental Procedures.

on 50 μM phospholipid vesicles with 5 mol % phosphatidylserine (Figure 4B).

Comparison of the Procoagulant Activities of Factor V₁ and Factor V₂. The experiments presented in the previous paragraphs show that under several conditions (*e.g.*, 20 μM phospholipid vesicles containing 10 mol % phosphatidylserine) the cofactor activities of factor Va₁ and factor Va₂ in prothrombin activation were approximately similar (Figure 4) while their sensitivities for APC were considerably different (Figures 2 and 3). This means that in a system, in which factor V is activated by factor Xa/thrombin and in which the factor Va that is formed is allowed to express its cofactor activity in prothrombin activation in the presence of APC and protein S, factor V₁ will exhibit a higher overall procoagulant activity (*i.e.*, will yield more thrombin) than factor V₂. This was confirmed by the experiment presented in Figure 5. In this experiment, equal amounts of factor V₁ or factor V₂ were activated by factor Xa and thrombin in reaction mixtures containing 12.5 μM vesicles with a phospholipid composition identical to that of activated platelet membranes (Bevers *et al.*, 1983), 1 μM prothrombin, 4 pM factor Xa, 2 nM APC, and 200 nM protein S at physiological ionic strength. In the case of factor V₁, this

indeed resulted in the formation of much more thrombin than in the case of factor V₂ (Figure 5). From the fact that the final level of thrombin formed with factor V₂ is ~14% of that of factor V₁, we conclude that the overall procoagulant activity of factor V₁ under these conditions was 7-fold higher than that of factor V₂.

DISCUSSION

Efficient inactivation of factor Va by APC and protein S requires the presence of negatively charged membranes (Walker *et al.*, 1979; Bakker *et al.*, 1992). The membrane presumably acts as a collecting device that binds factor Va, APC, and protein S and that promotes the assembly of an anticoagulant complex, thus accelerating factor Va inactivation by APC. In this paper, we show that one of the plasma forms of factor Va (*i.e.*, factor Va₁) is inactivated by APC at considerably lower rates than the other form of factor Va (factor Va₂). Factor Va₁ and factor Va₂ have different light-chain domains (Rosing *et al.*, 1993; Ortel *et al.*, 1994a,b) and bind with different affinities to negatively charged membranes (Rosing *et al.*, 1993). In agreement with this phenomenon, we observed that differences in the rates of APC-catalyzed inactivation of factors Va₁ and Va₂ are especially observed at low membrane concentrations and on membranes that contain less than 15 mol % of the anionic phospholipid phosphatidylserine. At high phospholipid and phosphatidylserine concentrations, factor Va₁ and factor Va₂ were inactivated at similar rates. Under these conditions, the number of available binding sites for factor Va on the membrane likely exceeds the dissociation constants of the factor Va–membrane complex of both factor Va₁ and factor Va₂. This results in a situation in which almost all factor Va₁ and Va₂ will be bound to the membrane and hence will be inactivated by APC at comparable rates.

Similar differences between APC-catalyzed inactivation of factor Va₁ and factor Va₂ were observed in the presence of the APC cofactor protein S. This indicates that, at the chosen reaction conditions, protein S does not affect the assembly of the factor Va–APC–membrane complex. Our data confirm that protein S specifically accelerates APC-catalyzed factor Va₁ and Va₂ inactivation by increasing the rate constant of cleavage at Arg³⁰⁶ (Rosing *et al.*, 1995).

Recently, we reported (Nicolaes *et al.*, 1995; Rosing *et al.*, 1995) a kinetic analysis of APC-catalyzed inactivation of a factor Va preparation that contained both factor Va₁ and factor Va₂. Careful assessment of cofactor activity in prothrombin activation showed that this preparation contained ~10% factor Va₁. Since the experiments reported in these papers were performed on 25 μM PS/PC (10/90, mol/mol) vesicles, factor Va₁ negligibly contributed to factor Va inactivation, and the given rate constants were actually those for factor Va₂.

Factor Va₁ and factor Va₂ also exhibited different cofactor activities in prothrombin activation. However, full expression of factor Va cofactor activity required much less phospholipid and phosphatidylserine than APC-catalyzed factor Va inactivation. This results in a situation in which at intermediate phospholipid conditions (*e.g.*, 20 μM phospholipid with 10 mol % phosphatidylserine) both forms of factor Va have the same cofactor activity in prothrombin activation but are inactivated by APC at considerably different rates (see also below).

When the properties of factor Va₁ and factor Va₂ are compared, it appears that their functional difference dimin-

ishes in the following order: binding affinity for negatively charged membranes (Rosing *et al.*, 1993), sensitivity for APC (this paper), and cofactor activity in prothrombin activation (Rosing *et al.*, 1993; this paper). This can be explained by the fact (1) that binding experiments yield true affinity differences of factor Va₁ and factor Va₂ for membranes independent of the phospholipid concentration, (2) that in APC-catalyzed factor Va inactivation this difference may be (partially) compensated at higher phospholipid concentrations (binding sites $\geq K_d$), and (3) that in prothrombinase measurements the presence of factor Xa (Rosing *et al.*, 1980; Nesheim *et al.*, 1980; Lindhout *et al.*, 1982) and prothrombin (Billy *et al.*, 1996) additionally contributes to the binding of factor Va to the membrane.

Our observation that much less phospholipid and phosphatidylserine are required for full expression of cofactor activity of factor Va in prothrombin activation than for regulation of factor Va cofactor activity by APC may have physiological implications. On membranes with 5–10 mol % phosphatidylserine, which likely simulate the *in vivo* situation (Beyers *et al.*, 1983), factor Va₁ and factor Va₂ have similar cofactor activities in prothrombin activation but exhibit considerable differences in their sensitivity for APC. This means that, in contrast to the expectation, the overall procoagulant activity of factor V₁ will exceed that of factor V₂ in reaction systems containing membranes with physiologic phospholipid compositions, factor V, factor Xa, prothrombin, APC, protein S, and traces of thrombin to initiate factor Va formation. The experiment presented in Figure 5, which was performed on membranes with the phospholipid composition of activated platelets, shows that this is indeed the case. The total amount of thrombin formed in the reaction system in which factor V was activated by factor Xa and thrombin and in which the factor Va formed was simultaneously allowed to express cofactor activity in prothrombin activation and to be down-regulated by APC and protein S was 7-fold higher with factor V₁ than with factor V₂. This shows that, due to differences in sensitivity for APC, the overall procoagulant activity of the form of factor V that has the lowest affinity for membranes (factor V₁) was at least 7 times higher than that of the form of factor V which has the highest membrane affinity (factor V₂).

In a recent paper, Zöller *et al.* (1994) reported that in approximately 5% of the APC-resistant families the Arg⁵⁰⁶-Gln mutation (factor V_{Leiden}), that is usually associated with APC resistance (Dahlbäck *et al.*, 1993; Bertina *et al.*, 1994), is not found and suggested that apart from this mutation other, as yet unknown, genetic defects may also cause inherited APC resistance. In addition, Zöller *et al.* (1994) described that heterozygous carriers of the factor V_{Leiden} mutation with a history of thrombosis had lower APC sensitivity ratios than heterozygous family members without thrombosis. It is conceivable, that individuals with low APC sensitivities in APTT-based assays may have ratios of circulating factor V₁ and factor V₂ that differ from that of normal individuals (normal plasma contains ~30% factor V₁; Rosing *et al.*, 1993). Based on the observations reported in the present paper, we are currently developing a method to quantitate the amounts of factor V₁ and factor V₂ in plasma. With such a procedure, we will investigate whether there is a relation between non-factor V_{Leiden} related APC resistance, plasma levels of factor V₁ and V₂, and a possible risk for venous thrombosis.

REFERENCES

- Bakker, H. M., Tans, G., Janssen-Claessen, T., Thomassen, M. C. L. G. D., Hemker, H. C., Griffin, J. H., & Rosing, J. (1992) *Eur. J. Biochem.* 208, 171–178.
- Bertina, R. M., Koeleman, B. P. C., Koster, T., Rosendaal, F. R., Dirven, R. J., de Ronde, H., van der Velden, P. A., & Reitsma, P. J. (1994) *Nature* 369, 64–67.
- Beyers, E. M., Comfurius, P., & Zwaal, R. F. A. (1983) *Biochim. Biophys. Acta* 736, 57–66.
- Billy, D., Willems, G. M., Hemker, H. C., & Lindhout, T. (1996) *J. Biol. Chem.* 270, 26883–26889.
- Bock, P. E., Craig, P. A., Olsen, S. T., & Singh, P. (1989) *Arch. Biochem. Biophys.* 273, 375–378.
- Chase, T., Jr., & Shaw, E. (1969) *Biochemistry* 8, 2212–2224.
- Comp, P. C., Nixon, R. R., Cooper, M. R., & Esmon, C. T. (1984) *J. Clin. Invest.* 74, 2082–2088.
- Dahlbäck, B., Carlsson, M., & Svensson, P. J. (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90, 1004–1008.
- DiScipio, R. G., & Davie, E. W. (1979) *Biochemistry* 18, 899–904.
- DiScipio, R. G., Hermanson, M. A., Yates, S. G., & Davie, E. W. (1977) *Biochemistry* 16, 698–706.
- Esmon, C. T. (1989) *J. Biol. Chem.* 264, 4743–4746.
- Griffin, J. H., Evatt, B., Zimmerman, T. S., & Kleiss, A. J. (1981) *J. Clin. Invest.* 68, 1370–1373.
- Gruber, A., Griffin, J. H., Harker, L., & Hanson, S. R. (1989) *Blood* 73, 639–642.
- Kalafatis, M., Rand, M. D., & Mann, K. G. (1994) *J. Biol. Chem.* 269, 31869–31880.
- Kalafatis, M., Bertina, R. M., Rand, M. D., & Mann, K. G. (1995) *J. Biol. Chem.* 270, 4054–4057.
- Lindhout, T., Govers-Riemslog, J. W. P., van de Waart, P., Hemker, H. C., & Rosing, J. (1982) *Biochemistry* 21, 5494–5502.
- Nesheim, M. E., Eid, S., & Mann, K. G. (1980) *J. Biol. Chem.* 256, 9874–9882.
- Nicolaes, G. A. F., Tans, G., Thomassen, M. C. L. G. D., Hemker, H. C., Varadi, K., Schwarz, H. P., & Rosing, J. (1995) *J. Biol. Chem.* 270, 21158–21166.
- Ortel, T. L., Yoo, L., Quin-Allen, M. A., & Kane, W. H. (1994a) *Blood* 84, 387a.
- Ortel, T. L., Quin-Allen, M. A., Keller, F. G., Peterson, J. A., Larocca, D., & Kane, W. H. (1994b) *J. Biol. Chem.* 269, 15898–15905.
- Pletcher, C. H., & Nelsestuen, G. L. (1982) *J. Biol. Chem.* 257, 5342–5345.
- Rosing, J., Tans, G., Govers-Riemslog, J. W. P., Zwaal, R. F. A., & Hemker, H. C. (1980) *J. Biol. Chem.* 255, 274–283.
- Rosing, J., Bakker, H. M., Thomassen, M. C. L. G. D., Hemker, H. C., & Tans, G. (1993) *J. Biol. Chem.* 268, 21130–21136.
- Rosing, J., Hoekema, L., Nicolaes, G. A. F., Thomassen, M. L. C. G. D., Hemker, H. C., Varadi, K., Schwarz, H. P., & Tans, G. (1995) *J. Biol. Chem.* 270, 27852–27858.
- Sala, N., Owen, W. G., & Collen, D. (1984) *Blood* 63, 671–675.
- Schwarz, H. P., Fisher, M., Hopmeier, P., Batard, M. A., & Griffin, J. (1984) *Blood* 64, 1297–1300.
- Smith, R. L. (1973) *J. Biol. Chem.* 248, 2418–2423.
- Solymoss, S., Tucker, M. M., & Tracy, P. B. (1988) *J. Biol. Chem.* 263, 14884–14890.
- Tans, G., Rosing, J., Thomassen, M. C. L. G. D., Heeb, M. J., Zwaal, R. F. A., & Griffin, J. H. (1991a) *Blood* 77, 2641–2648.
- Tans, G., Janssen-Claessen, T., Hemker, H. C., Zwaal, R. F. A., & Rosing, J. (1991b) *J. Biol. Chem.* 266, 21864–21873.
- Walker, F. J. (1980) *J. Biol. Chem.* 255, 5521–5524.
- Walker, F. J., Sexton, P. W., & Esmon, C. T. (1979) *Biochim. Biophys. Acta* 571, 333–342.
- Zöller, B., Svensson, P. J., He, X., & Dahlbäck, B. (1994) *J. Clin. Invest.* 94, 2521–2524.